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APPENDIX B
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Neville *et al.*
Serial No. 09/389,565
Filed: September 3, 1999
For: "AN IMMUNOTOXIN WITH IN VIVO T CELL SUPPRESSANT ACTIVITY AND METHODS OF USE"

Examiner: G.R. Ewoldt, PhD.
Art Unit: 1644

DECLARATION OF DAVID M. NEVILLE, JR. UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington DC 20231

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May 1, 2002

Examiner Ewoldt:

I, David M. Neville, Jr., a citizen of the United States, residing at 9624 Parkwood Drive, Bethesda, Maryland 20814, declare that:

1. A fusion immunotoxin constructed of the first 390 residues of diphtheria toxin followed by the sFv of the anti-CD3ε antibody of UCHT1 has surprising and unexpected properties of *in vivo* T cell depletion of resting T cells, activated T cells and malignant T cells. These surprising and unexpected properties arise from characteristics of the sFv moiety of UCHT1 that are not present in most other sFv constructs

of other anti-CD3 antibodies and also arise from the unique synergy between DT390 and the CD3ε epitope.

2. In my laboratory and in the laboratories of our collaborators, fusion immunotoxins of three additional anti-CD3 sFvs have been made and tested. In each case, the truncated DT moiety consisted of either DT389 plus a short spacer or DT390. The sFvs constructs were made using a V_L-L-V_H where the linker (L) was either $(G_4S)_3$ or $(G_3S)_4$.

3. Fusion immunotoxins made with sFvs of FN18 (anti-rhesus CD3ε), SP-34 (anti human/rhesus CD3) and CHRIS-7 (anti human/rhesus CD3) showed a 2-3 log loss of potency when compared to DT390sFv or DT389sFv of UCHT1. Each of these parental antibodies (FN18, SP-34, CHRIS-7, and UCHT1) has approximately the same affinity when assayed by FACS analysis on monkey or human T cells expressing CD3. A chemical conjugate of the SP34 antibody with a DT binding site mutant CRM9 exhibits only a 0.5 log loss of toxicity compared with the analogous UCHT1-CRM9 conjugate. These data suggest that the non-predictable features lie in the variable sFv properties of UCHT1.

4. Research was performed in my laboratory to assess the binding of DT390sFv constructs to the appropriate T cells by FACS competition binding studies. See Thompson et al., 2001, which is attached hereto as Exhibit A. The best sFv derived from UCHT1 shows a decrease in binding of 1.8 logs compared to its parental antibody. Despite the reduced binding, the specific toxicity of the DT390sFv towards T cells is equal to that of the chemical conjugate of UCHT1-CRM9. The FN18 sFv, in contrast, shows a greater than 2 log loss in binding (Ma et al., 1997, which is attached hereto as Exhibit B) but reduced T cell toxicity as compared to the chemical conjugate of UCHT1-CRM9.

5. This loss of binding in the various sFvs appears to be caused by steric hindrance from the large toxin moiety amino-terminal to the sFv

(Thompson et al., 2001, Tables I and II (Exhibit A)). The unpredictable differences between various sFvs with respect to specific binding and specific T cell toxicity when present in fusion immunotoxins may reflect the varying stability of the sFv to maintain its structure in the face of neighboring steric hindrance, but the state of the art at the time of the invention, and even now, is such that one skilled in the art cannot predict the relative success of a given sFv moiety when placed carboxy-terminal to DT390.

6. DT390sFv(UCHT1) is a superior anti-T cell immunotoxin but the reason for this superiority has not yet been fully elucidated. DT390sFv(UCHT1) produces profound (>1.5 logs) *in vivo* depletion of resting T cells (Thompson et al., 2001, Fig. 2 (Exhibit A)), whereas similar DT390sFv immunotoxins fail to produce a comparable depletion.

7. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or document or any patent issuing therefrom.

5/03/02
Date

David M. Neville, Jr.

Exhibit A

Thompson, J., Stavrou, S., Weetall, M., Hexam, M., Digan, M.E., Wang, Z., Woo, J.H. Yu, Y., Mathias, A., Liu, Y.Y., Ma, S., Gordienko, I., Lake, P. and Neville DM Jr., Improved Binding of a Bivalent Single-Chain Immunotoxin Results in Increased Efficacy for *In Vivo* T-cell Depletion. Protein Engineering 14(12):1035-1041, 2001.

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Improved binding of a bivalent single-chain immunotoxin results in increased efficacy for *in vivo* T-cell depletion

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Anti-CD3 immunotoxins exhibit considerable promise for the induction of transplantation tolerance in pre-clinical large animal models. Recently an anti-human anti-CD3ε single-chain immunotoxin based on truncated diphtheria toxin has been described that can be expressed in CHO cells that have been mutated to diphtheria toxin resistance. After the two toxin glycosylation sites were removed, the bioactivity of the expressed immunotoxin was nearly equal to that of the chemically conjugated immunotoxin. This immunotoxin, A-dmDT390-sFv, contains diphtheria toxin to residue 390 at the N-terminus followed by VL and VH domains of antibody UCHT1 linked by a $(G_4S)_3$ spacer (sFv). Surprisingly, we now report that this immunotoxin is severely compromised in its binding affinity toward $CD3^+$ cells as compared with the intact parental UCHT1 antibody, the UCHT1 Fab fragment or the engineered UCHT1 sFv domain alone. Binding was increased 7-fold by adding an additional identical sFv domain to the immunotoxin generating a divalent construct, A-dmDT390-bisFv (G_4S). *In vitro* potency increased 10-fold over the chemically conjugated immunotoxin, UCHT1–CRM9 and the monovalent A-dmDT390-sFv. The *in vivo* potency of the genetically engineered immunotoxins was assayed in the transgenic heterozygote mouse, tgε 600, in which the T-cells express human CD3ε as well as murine CD3ε. T-cell depletion in the spleen and lymph node observed with the divalent construct was increased 9- and 34-fold, respectively, compared with the monovalent construct. The additional sFv domain appears partially to compensate for steric hindrance of immunotoxin binding due to the large N-terminal toxin domain.

Keywords: CD3/depletion/divalent/immunotoxin/T cell/transplantation

Introduction

Immunotoxins are protein toxins that have undergone an alteration in cell specificity by replacement of the toxin receptor-binding domain with an alternative receptor-binding domain. As originally formulated, enzymatically active toxin A chain of diphtheria toxin was coupled to the alternate receptor binding domain of placental lactogen (Chang *et al.*, 1977). This and later work showed that the A chains of most toxins lacked efficient membrane protein translocation function and potent immunotoxins required a portion of the toxin B chain to aid the translocation process (Youle and Neville, 1982; Colombatti *et al.*, 1986; Williams *et al.*, 1987; Johnson *et al.*, 1988; Siegall *et al.*, 1989). Structural and genetic studies identified the toxin domains and specific sequences involved primarily with binding as opposed to translocation (Choe *et al.*, 1992). The advent of recombinant methodology led to the construction of immunotoxin fusion proteins with domains that bound to cells using targeting molecules such as hormones, cytokines or recombinant antibody derivatives (Williams *et al.*, 1987; Siegall *et al.*, 1989; Kuan and Pastan, 1996). However, immunotoxins that can kill 2–3 logs of target cells *in vivo* without high systemic toxicity have been elusive. Often, promising *in vitro* studies or *in vivo* xenograft models with cells exhibiting very high levels of receptor expression have failed to translate into good clinical therapies. Besides receptor number, other major variables affecting potency include down-stream events in the intoxication pathway such as toxin processing reactions and receptor-mediated cell routing. Both of these vary with cell type and receptor type (Neville *et al.*, 1989; Francisco *et al.*, 1996).

The T cell receptor protein CD3ε efficiently mediates the entry of diphtheria toxin-based immunotoxins (Neville *et al.*, 1989). In monkeys, reduction of 2–3 logs of lymph node T cells is achieved using a chemical conjugate of anti-rhesus CD3 antibody and CRM9, a diphtheria toxin binding-site mutant (Neville *et al.*, 1996; Hu *et al.*, 1997). Long-term tolerance to xenograft pancreatic islet transplants and renal allograft transplants have been described using this material (Contreras *et al.*, 2000; Thomas *et al.*, 2000). We have described a monovalent recombinant form of this conjugate and its anti-human counterpart, DT390-sFv (UCHT1), that has reduced sensitivity to anti-DT neutralizing antibodies (Thompson *et al.*, 1995; Ma *et al.*, 1997). The sFv moiety in this type of immunotoxin is restricted to the toxin C-terminus because the N-terminal toxin A chain can accept only a limited number of foreign residues before its enzymatic properties are compromised (Madshus *et al.*, 1992).

This paper reports an optimized derivative DT390-sFv (UCHT1) immunotoxin. Our focus has been on improving the binding affinity of this recombinant immunotoxin. It appeared to us that this could most easily be accomplished by increasing the valency of the immunotoxin to reflect the divalency of the parental antibody. Our work has been patterned on previous efforts to form multivalent recombinant antibody derivatives (Holliger *et al.*, 1993; Shu *et al.*, 1993; Whitlow *et al.*, 1993; Kipriyanov *et al.*, 1994; Hu *et al.*, 1996), either through single-chain tandem sFv groups or through heavy chain disulfide

bound dimeric structures, the so-called minibodies. We therefore made a systematic investigation of the binding of anti-CD3ε antibody fragments and recombinant derivatives to CD3ε expressing cells and the immunotoxins so derived. Linker size and placement were also investigated. In order to judge the *in vivo* potency of our optimized immunotoxin, we used a transgenic mouse that expresses human CD3ε (Wang *et al.*, 1998), thereby acquiring pre-clinical data but avoiding the use of large non-human primates. Our data suggest that this newly optimized molecule has valuable *in vivo* potential for the treatment of T cell tumors as well as induction of tolerance for transplantation.

Materials and methods

Monovalent immunotoxin construction

The construction of DT390-sFv has been described (Thompson *et al.*, 1995). This construct contains the first 390 amino acids of native DT, including the secretory signal peptide, upstream of the UCHT1 sFv [in the variable light (V_L) to variable heavy (V_H) configuration]. The V_L–V_H–linker is $(G_4S)_3$. A variant monovalent construct, M-DT389-sFv, was also produced (Hexham *et al.*). This construct was expressed in *Escherichia coli* without a signal peptide and was refolded and purified from cytoplasmic inclusion bodies. This construct encoded an N-terminal methionine residue. In addition to having one less DT residue, this construct also contained a six-residue flexible linker (ASAGGS) between the DT moiety and the sFv moiety and the sequence of the V_L–V_H–linker was changed to $(G_3S)_4$, see Fig. 1.

Bivalent single-chain immunotoxin construction

The generation of the bisFv construct was completed in two separate steps. In the first step, the 5′ end of the UCHT1-sFv (V_L) was modified by polymerase chain reaction (PCR) to contain part of the linker sequence (the 3′ end of the linker was added to the 5′ end of the sFv). Additionally, the 3′ end of the UCHT1-sFv (V_H) was modified to incorporate the 5′end of the linker sequence. In the second step, the two sFv DNA sequences (V_L–V_H–linker and linker–V_L–V_H) were verified to encode the appropriate amino acid sequences and subcloned together (V_L–V_H–linker–V_L–V_H) using the unique *Bam*HI restriction enzyme site. The bisFv sequence was then subcloned downstream of the DT390 sequence in pET15b to generate DT390-bisFv (G4S).

A similar scheme was used to create a bisFv construct using the CHB1 linker (Mallender and Voss, 1994), between the two sFv domains. For the CHB1 linker, the two sFv DNA sequences (V_L–V_H–linker and linker–V_L–V_H) were verified to encode the appropriate amino acid sequences and subcloned together (V_L–V_H–linker–V_L–V_H) using the unique *Xho*I restriction enzyme site. The bisFv sequence was then subcloned downstream of the DT390 sequence in pET-15b to generate DT390-bisFv (CHB1).

Construction, expression and quantification of UCHT1 derivatives

The Fab fragment of UCHT1 was prepared by papain digestion using cross-linked agarose–papain (Pierce) following the manufacturer's directions. Contaminating Fc fragments and undigested UCHT1 were removed by absorption on to protein A Sepharose (Pierce). Fab was quantified by size-exclusion HPLC (GF-250 Zorbax) and UV absorption and integration using a UCHT1 standard and a correction for MW reduction.

The sFv and bisFv of UCHT1 were amplified by PCR. The template was DT390-bisFv (G4S) (see above) and the primers were chosen from the 5′ end of V_L and the 3′ end of V_H. The PCR products were checked by gel electrophoresis and the bands of sFv and bisFv of UCHT1 were cut out and extracted with a QIAquik Gel Extraction Kit (Qiagen, Chatsworth, CA). The extracted DNA was digested with *Nde*I and *Eco*RI and cloned into expression vector pET17b (Novagen) at *Nde*I and *Eco*RI sites. These were transformed into *E.coli* strain: BL21 (DE3)pLysS competent cells (Novagen). A single colony was cultured overnight. The overnight culture was re-cultured for 3–4 h with fresh medium to reach an OD_{600} of 0.6, after which IPTG, 1 mM, was added to induce expression. The bacterial pellet was harvested by centrifugation. Inclusion bodies were prepared by the method of Buchner *et al.* (Buchner *et al.*, 1992) and solubilization of inclusion bodies and refolding of the protein were performed according to Vallera *et al.* (Vallera *et al.*, 1996).

Minibody construction and expression

The [sFv(UCHT1)-H-γCH3-h]2 minibody was constructed by PCR overlap extension. This construct was patterned on the flex minibody described by Hu *et al.* (Hu *et al.*, 1996). The sFv (UCHT1) template was DT390-sFv (see above). In the minibody, the sFv region is followed by the hinge region of human IgG1 (residue 216 to residue 229). These residues were amplified from a plasmid containing human IgG1 heavy chain (supplied by Dr Syed Kashmiri, National Cancer Institute). In the hinge, residue C220 was changed to P (Shu *et al.*, 1993) leaving C226 and C229 residues to form the interchain disulfide dimer. Between the hinge and γCH3, a flexible spacer, $(G_3S_2)_2$, was inserted following the construction of Hu *et al* (Hu *et al.*, 1996). A histidine (h) tag of six residues was added at the C-terminus of γCH3 and a murine kappa signal peptide, MSVPTQVLGLLLLWLTDARC, was placed 5′ to the sFv (Xiang, 1992). This gene was cloned into pBacPAK8 (Clontech, Palo Alto, CA) and expressed in S9 cells at a level of 5 μg/ml as quantified by Coomassie Brilliant Blue staining using UCHT1 as a standard. Although the sFv of UCHT1 does not bind to Protein L Plus (Pierce) at pH 8.0 in PBS, it does bind in the presence of high salt. One volume of culture medium was mixed with 1.5 volumes of 2.5 M glycine–5 M NaCl, pH 9.0, and applied to a 1 ml column volume of Protein-L plus equilibrated with 1.5 M glycine–3 M NaCl, pH 9.0. The column was washed with two column volumes of application buffer and eluted with 4 ml of 0.25 M glycine–HCl, pH 2.5. The divalent construct was quantified by Coomassie Brilliant Blue staining of SDS gels using a Fab (UCHT1) standard.

The [sFv(UCHT1)-μCH2-h]2 minibody construct was made by using PCR amplification, from previously cloned single-chain human IgM antibody construct (Ma *et al.*, 1996). The sFv(UCHT1)-μCH2-h construct was amplified by using 5′ sFv and 3′ CH2 primers. A six histidine residue tag was introduced to the 3′ end of the CH2 domain. Following purification of the amplified sFv(UCHT1)-μCH2-h fragment by gel elution and digestion with *Eco*RI and *Not*I, it was inserted between the *Eco*RI and *Not*I sites of pET17b vector (Novagen). *E.coli* XL-1 Blue strain was used for all plasmid constructions. For expression in *Pichia pastoris*, pPICZα (Invitrogen, Carlsbad, CA) was used as the *Pichia* expression vector. The DNA sequence was confirmed by sequencing. KM71 was used as the host strain (Invitrogen). Maximum secretion of divalent

anti-CD3 minibody could be obtained at 4 days after methanol induction in 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% methanol plus 1% casamino acids to retard proteolysis. Western blots from non-reducing and reducing gels probed with polyclonal anti human IgM (Life Technologies, Bethesda, MD) confirmed the disulfide dimer, which accounted for 75% of the secreted material. Partial purification was achieved by absorbing contaminating proteins on DEAE Sepharose at pH 8.5 and applying the flow-through to Protein L agarose (Pierce) in the presence of 1.5 M glycine and 3 M NaCl, pH 8.9, and eluting with PBS diluted 1:3 in water. The divalent construct was quantified by Coomassie Brilliant Blue staining of SDS gels using a Fab (UCHT1) standard.

Construction, expression and quantification of HSA-sFv(UCHT1)

The gene for the HSA-sFv(UCHT1) fusion construct codes from 5′ to 3′ the 609 residues of human serum albumin precursor variant A followed by a six-residue flexible linker (ASAGGS) and then the sFv moiety of sFv (UCHT1) used in DT389-sFv (see above). The fusion was performed by PCR overlap extension. This gene was cloned into the pHILD2 vector (Invitrogen) under the AOX1 promoter and expressed as a secreted form in *Pichia pastoris* GS115. HSA-sFv(UCHT1) was purified from the supernatant by ammonium sulfate precipitation, followed by cation-exchange chromatography on a Bio-Rad S2 column at pH 6.0, where the protein was in the unbound fraction. HSA-sFv(UCHT1) was quantified by Lowry protein assay using an HSA standard.

Expression of other immunotoxins

The production of recombinant immunotoxins such as dmDT390-sFv(His6) from stably transfected DT resistant CHO cell lines by means of the pSRα-neo vector has been described (Liu *et al.*, 2000). The notation dm refers to the double mutation removing the potential *N*-glycosylation sites at positions 16–18 in the DT A chain and positions 235–237 in the DT B chain. The signal peptide used in CHO expression contained an additional terminal alanine to optimize the cleavage process at an Ala–Ala junction and therefore added an Ala residue to the DT N-terminus. This new construct is now called A-dmDT390-sFv (after genetic removal of the C terminal His 6 tag). For CHO cell expression of the bivalent single-chain construct, DT390 was removed at the *Nco*I site from the DT390sFv plasmid for *E.coli* expression and replaced by sp-dmDT390 yielding on expression A-dmDT390-bisFv (G4S). The production of the disulfide-linked immunotoxins (A-DT390-sFv-H-γCH3-h)2 and (A-DT390-sFv-μCH2-h)2 was performed in CHO cells as described above, except that the glycosylation sites were not removed by mutation. These constructs were treated with *N*-glycosidase prior to toxicity assays under conditions that removed all detectable glycosylated forms (Liu *et al.*, 2000). M-DT389-sFv was expressed in *E.coli* and purified from cytoplasmic inclusion bodies as indicated above. Routine quantification was by Lowry protein assay calibrated by mass spectrometry.

Protein synthesis inhibition assay

Inhibition assays were performed as described previously (Neville *et al.*, 1989). Briefly, various concentrations of immunotoxin were incubated with Jurkat cells (1×10^4 cells/well of a 96-well plate) for 20 h. A 1 h pulse of [^{3}H]leucine (4.5 μC/ml) was given before cells were collected on filters with a Skatron harvester (Skatron, Sterling, VA). Samples were counted in a scintillation counter. Each experiment was performed in quadruplicate. Results were calculated into a mean value and recorded as a percentage of control cells.

Table I. Binding of UCHT1 derivatives relative to UCHT1 on Jurkat cells

Derivative	Source	Relative binding[a]
UCHT1	Hybridoma	1.0
Fab	Hybridoma + papain	0.6 ± 0.2
Minibody (sFv-H-γCH3-h)2	SF-9 cells	1.2 ± 0.3
Minibody (sFv -μCH2-h)2	*Pichia*	1.1 ± 0.5
SFv	*E.coli*	0.32 ± 0.05
bisFv (sFv-L-sFv)	*E.coli*	1.5 ± 0.1
HSA-sFv	*Pichia*	0.009 ± 0.001

[a]Concentration of UCHT1/concentration of derivative giving equal MCF′ values after competing UCHT1–FITC tracer bound to Jurkat cells. Most values are interpolated from MCF′ versus log concentration plots and are presented as means ± SD (see Materials and methods).

Recombinant antibody and immunotoxin affinity estimation by FACS analysis

The anti-human anti-CD3ε antibody, UCHT1, kindly provided by Dr P.Beverley, Imperial Cancer Research Fund, was derivatized with FITC at 5 mol/mol Ab. This was used as a tracer at 5×10^{-9} M and competed with various concentrations of UCHT1 and test materials in parallel binding to Jurkat cells or in some cases human PBMC at 4°C for 30 min before washing by centrifugation and being subjected to FACS gating on live cells with propidium iodide. Mean channel fluorescent values (MCF′) were corrected by subtracting an appropriate FITC non-binding isotype control. The binding affinity of a ligand relative to UCHT1 was calculated by dividing the concentration of UCHT1 by the concentration of ligand that gave equal MCF′ values. When the observed MCF′ were not equal, the UCHT1 concentration required to match a ligand MCF′ value was interpolated from plots of MCF′ versus log concentration of ligand and UCHT1. These plots were roughly linear between tracer displacement values between 10% and 90%; however, the curves were not uniformly parallel. Interpolations were performed at low, medium and high displacement values when possible and the means and SDs were calculated with values of n ranging between 2 and 6. In several cases the relative affinity of the chemical conjugate UCHT1–CRM9 was compared with that of a recombinant immunotoxin by observing the MCF′ values of polyclonal FITC-labeled anti-DT over a range of concentrations. The second antibody method gave comparable results to the competition method when the difference in affinity of UCHT1 and UCHT1–CRM9 was included in the calculation.

T cell depletion in tgε600$^{+/-}$ mice

tgε600 homozygous mice were obtained from Dr Cox Terhorst (Beth Israel Hospital, Harvard Medical School, Boston, MA). In this work the heterozygote strain of the mouse, tgε600$^{+/-}$, was used (F1 of tgε 600×C57BL/6J). These mice contain three copies of the complete human CD3ε gene under the transcriptional regulation of their endogenous promoters and enhancers (Wang *et al.*, 1998). A twice per day experimental dosing regimen was chosen because the monovalent immunotoxin was observed to have a short half-life in mice (<6 h). Accordingly, tgε600$^{+/-}$ were treated with immunotoxin twice daily for 4 days by tail vein administration using 0.1% murine serum albumin as a vehicle to prevent adsorption to plastic

Table II. Relative binding and toxicity of UCHT1-based immunotoxins on Jurkat cells

Construct	Source	Relative binding[a]	Relative toxicity[b]
UCHT1–CRM9	Conjugation	0.310 ± 0.090	1.0
Fab-CRM9	Conjugation	0.190 ± 0.160[c]	0.3
M-DT389-sFv	*E.coli*	0.015 ± 0.003	0.9 ± 0.1
A-dmDT390-sFv	CHO cells	0.014 ± 0.003	1.3 ± 0.4
A-dmDT390-bisFv	CHO cells	0.094 ± 0.04	13 ± 11
(DT390-sFv-H-γCH3-h)2	CHO cells	<0.01	0.01[d]
(DT390-sFv-μCH2-h)2	CHO cells	<0.01	0.05[d]

[a]Relative binding calculated as in Table I.
[b]IC_{50} of construct/IC_{50} of UCHT1–CRM9, reported as means when SD is given.
[c]Binding assayed by anti-DT FITC Ab rather than competition.
[d]After deglycosylation with pNGase (see Materials and methods).

surfaces. Approximately 16 h after the final treatment, the lymph nodes and spleen were removed and single cell suspensions were prepared from individual mice. Total cells were counted by chamber counts. The percentage of CD3 positive cells was assessed by two-color FACS analysis using a FITC-conjugated anti-human CD3 antibody (UCHT1–FITC) to measure human CD3 expression, phycoerythrin (PE)-conjugated anti-mouse CD3 antibody (500A2–PE) to measure expression of mouse CD3 and the appropriately stained isotype controls. Viaprobe (Pharmingen, San Diego, CA) was used to gate on the live cells. Gates were set to count the fraction of double positive (DP) events ($huCD3^+muCD3^+$) for the isotype controls in non-treated animals and the fraction of DP events in treated animals. The fraction of DP isotype controls was subtracted from the fraction of DP events in treated animals and the result, DP′, multiplied by the total cell count. This was subtracted from the DP′ cells in control animals. The result was divided by the DP′ cells in control animals to give the fractional depletion in DP cells at each immunotoxin concentration. (In non-treated animals 98–99% of the lymph node and spleen T cells were DP cells, the remaining being single positive $muCD3^+$.) Mean fractional lymph node and spleen T cell depletion values were calculated for each immunotoxin concentration. The data were fitted by probit analysis using a log transformation of the concentration scale using SPSS software (SPSS, Chicago, IL). In probit transformation instead of regressing the actual proportion responding to the values of the stimuli, each of the observed proportions is replaced with the value of the standard normal curve below which the observed proportion of the area is found (Finney, 1978). Data points for monovalent and divalent immunotoxin were fitted alone or together to yield parallel curves with one regression coefficient.

Results

Bivalent immunotoxin has increased binding

One potential way to increase the toxicity of the immunotoxin is to increase the binding of the molecule to the target, i.e. increase the affinity. To increase the binding characteristics of the immunotoxin, we prepared a variety of recombinant bivalent anti-CD3ε constructs and estimated their relative affinity compared to the parental antibody, UCHT1, and the monovalent Fab fragment of UCHT1. These studies were done to help us optimize the binding moiety of recombinant anti-CD3 immunotoxin. The mean relative binding values of the UCHT1 single-chain derivatives, lacking the DT moiety of the immunotoxin, were tested on Jurkat cells using the FACS-based assay (Table I). Because plots of corrected mean channel fluorescence versus log concentration of competing UCHT1 or test ligands were not always parallel, comparisons were made at high, medium and low values of UCHT1–FITC displacement and the means and SDs were calculated. High SD values reflect relatively non-parallel curves.

The bivalent recombinant minibodies that were expressed in eukaryotic cells were secreted as disulfide dimers and had affinities equal to the parental divalent antibody. This was the case for the minibody that contained the hinge region and the γCH3 domain of human IgG1 as well as the minibody based on the human μCH2 domain. However, the monovalent sFv refolded from *E.coli* showed only half the affinity of the monovalent Fab fragment. The bisFv also refolded from *E.coli* was very avid and bound 1.5-fold better than the parental antibody. A significant finding was the fact that inserting a large protein domain at the N-terminus of the sFv domain, as in human serum albumin-sFv construct, decreased the binding by 100-fold compared with the parental antibody and decreased by 35-fold from the sFv alone. Indeed, addition of the DT moiety reduced the relative affinity of all immunotoxins tested (Table II). The monovalent recombinant immunotoxins exhibit a large drop in affinity compared with their free sFv counterparts, similar to that seen by fusing human serum albumin on to the sFv N-terminus.

Bivalent single-chain immunotoxin has increased toxicity

To determine whether the observed increase in affinity of the bivalent constructs does increase the toxicity of immunotoxins, the DT390 moiety was added to each bivalent construct. The bivalent immunotoxins were tested for their toxicity on Jurkat cells in a 20 h protein synthesis inhibition assay (Table II). The disulfide-linked dimer immunotoxins were relatively non-toxic. The monovalent immunotoxins had similar activity to UCHT1–CRM9 and an ~3-fold increase in toxicity compared with the monovalent Fab-CRM9. There was no significant difference between the M-DT389-sFv that was produced in *E.coli* and refolded from inclusion bodies and the A-dmDT390-sFv construct that was secreted from CHO cells. However, it should be noted that the bacterial construct exhibited <3% nicking at the furin-sensitive bond between the A and B toxin chains, while the CHO material exhibited variable degrees of nicking from various preparations that ranged from 20 to 50%. Higher fractions of nicked material increased toxicity in Jurkat assays by a factor of 1.5 (data not shown). A bivalent construct utilizing a different linker sequence between the two sFv domains (Mallender and Voss, 1994), DT390-bisFv (CHB1), demonstrated toxicity equivalent to DT390-sFv (data not

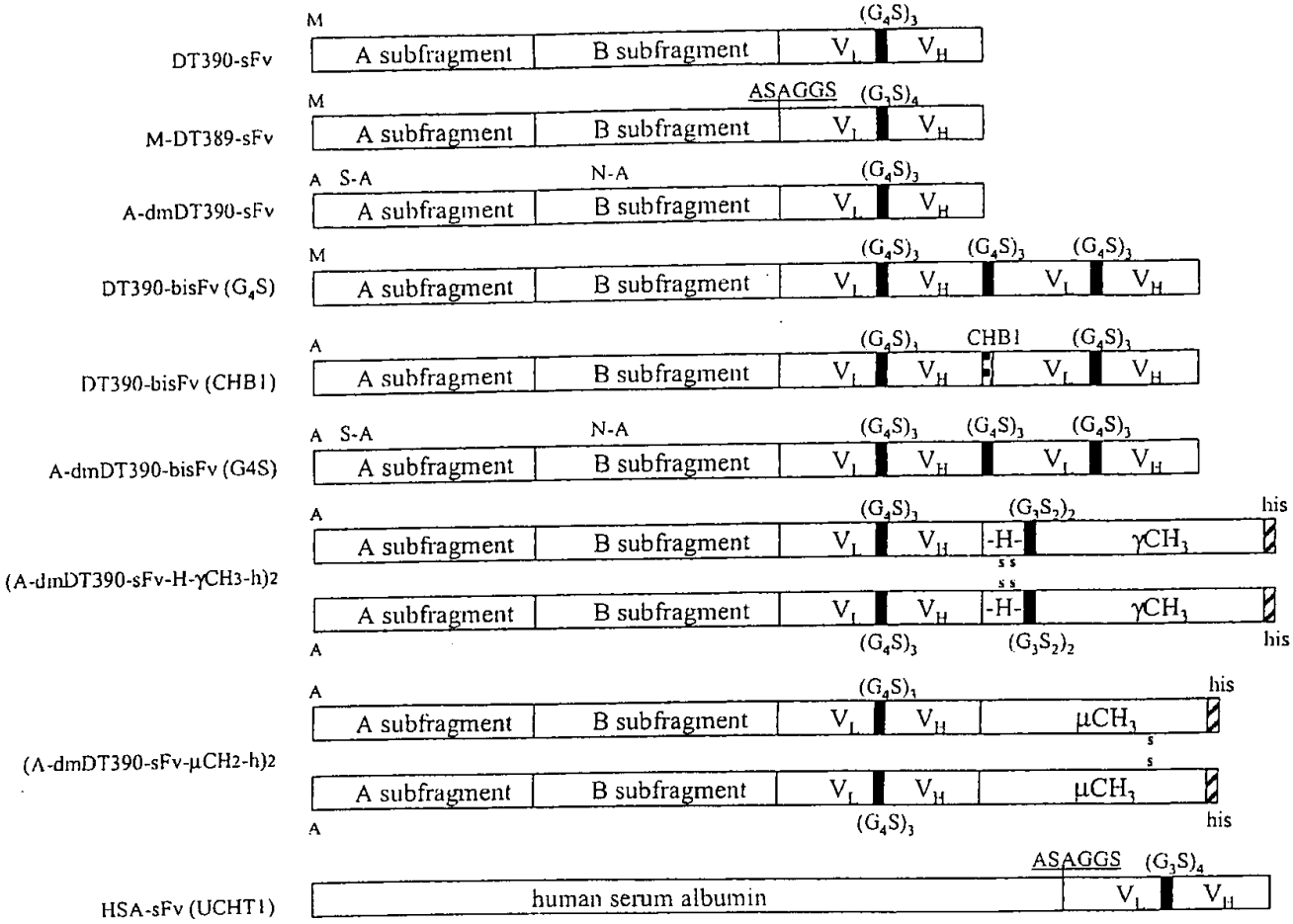

Fig. 1. Schematic representation of constructs. The immunotoxin constructs referred to in Table II are illustrated, together with other constructs mentioned in the text. To determine the schematic UCHT1 derivatives used for the binding assay described in Table I, simply remove the DT390 moiety (A subfragment and B subfragment) from the representation above. The HSA-sFv (UCHT1) used in Table I is also shown since it has no DT constituent. The CHB1 linker described by Malleneder and Voss (Mallender and Voss, 1994) contains the amino acid sequence PGGNRGTTRPATSGSSPGPTNSHY in the indicated linker space. The (A-dmDT390-sFv-H-γCH3)2 and (A-dmDT390-sFv-μCH2)2 are secreted and purified as dimers which are bound by disulfide bridges (S–S) as indicated. The histidine tag (his) is shown. The double mutant (dm) amino acid changes are indicated (18S-A and 235N-A).

shown). On the other hand, the DT390-bisFv (G4S) construct showed increased toxicity compared with DT390-sFv. The *in vitro* translated DT390-sFv is ~1.5 logs less toxic than UCHT1–CRM9 whereas purified DT390-sFv has similar toxicity to the chemical conjugate (Liu *et al.*, 2000). The relative toxicity between single-chain immunotoxins from the *in vitro* coupled transcription and translation has always predicted the relative toxicity of purified protein (data not shown).

Bivalent immunotoxin depletes human CD3 positive T-cells in tgε600$^{+/-}$ mice

To demonstrate the *in vivo* significance of the increased activity of the bivalent immunotoxin, we compared the ability of the bivalent and monovalent immunotoxins to deplete human CD3 positive T-cells in tgε600$^{+/-}$ mice. Increasing concentrations of immunotoxin were administered twice daily for 4 days. Spleens (Figure 2A) and lymph nodes (Figure 2B) from 3–9 animals were used to generate the means for each concentration. In both spleen and lymph node the mean depletion values from divalent immunotoxin are shifted to the left, indicating higher potency. The curves were fitted by probit analysis. The regression model is transformed $Pi = A + B\log Xi$, where Pi is the observed proportion responding at dose $\log Xi$ and B is the regression coefficient. The regression coefficient is related to the fractional depletion F by the empirical equation $F = X^B/X^B + (IC_{50})^B$. The divalent fitted curves are the solid lines. For the spleen fit, 54 cases were available for monovalent and 39 cases for divalent immunotoxin and the fits in Figure 2A were performed individually. The regression coefficients shown in Table III are nearly identical and the curves are nearly parallel. When both cases are fitted together (93 cases), the changes are minimal. The divalent immunotoxin is nine times more potent than the monovalent. The significance is high (Table III). In the lymph node fit shown in Figure 2B, both monovalent and divalent were fitted together (89 cases). The curves are more shallow compared with the spleen curves. This result is influenced by the lowest concentration mean value of the divalent immunotoxin, which has a mean value of 0.4 compared with 0 in the spleen. In the lymph node the divalent immunotoxin appears 34-fold more potent than the monovalent immunotoxin based on the probit model.

Discussion

Previously, we reported the construction of a single-chain immunotoxin made with a truncated DT toxin moiety, DT390-

Table III. Increased T-cell depletion in spleen and lymph node induced by divalent recombinant anti-CD3 immunotoxin in tgε600 mice

Measurement	IC_{50} (pmol/kg); 95% confidence limits	Regression coefficient; standard error	Relative median potency bivalent/ monovalent; 95% confidence limits
LN bivalent[a]	2.58; 2.46, 2.70	0.913; 0.0080	0.029; 0.026, 0.032
LN monovalent[a]	89.1; 86.3, 92.1		
Spleen bivalent[a]	5.86; 5.65, 6.08	1.090; 0.0075	0.110; 0.100, 0.120
Spleen monovalent[a]	53.1; 51.4, 54.7		
LN bivalent	1.64; 1.53, 1.75	0.619; 0.0008	
Spleen bivalent	6.26; 6.04, 6.49	1.180; 0.0100	
LN monovalent	107; 105, 110	1.330; 0.0120	
Spleen monovalent	47.9; 46.0, 49.8	0.993; 0.0100	

[a]Monovalent and bivalent data are fitted together generating parallel curves and a single regression coefficient.

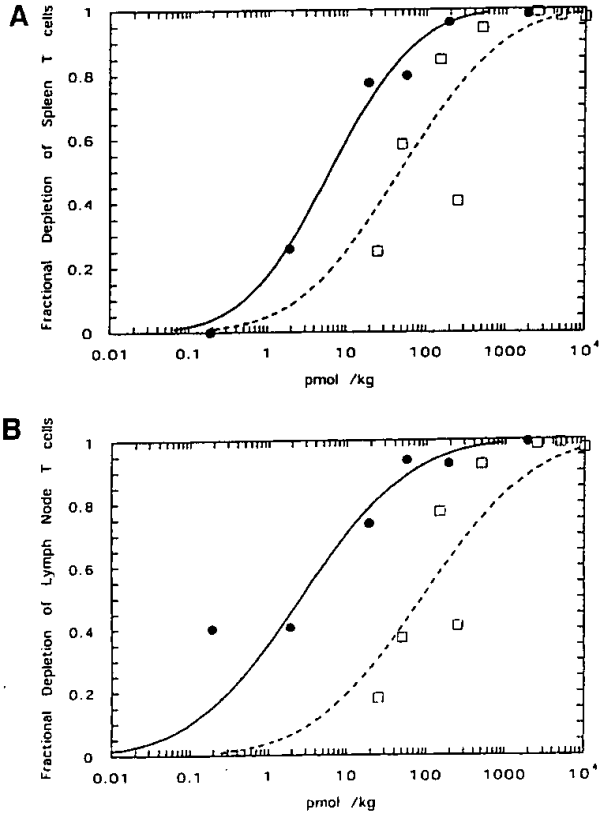

Fig. 2. Fractional *in vivo* T cell depletion induced by varying concentrations of monovalent and divalent single-chain immunotoxins in Tgε600 heterozygote mice in spleen and lymph node. (**A**) Depletion of double positive splenic T cells (human $CD3\varepsilon^+$, murine $CD3\varepsilon^-$) assayed by FACS. Open symbols are the mean values for the monovalent and closed symbols are the mean values for divalent immunotoxin. Dashed and solid lines are the probit model fits respectively performed individually. See Table III for probit-derived data. (**B**) As in (A) except done on lymph nodes. In this case all data points are fitted together generating a single regression coefficient for both immunotoxins. The listed dose is the total dose given over 4 days.

sFv, which was almost as toxic as a chemical conjugate made with the full length DT and intact antibody (Thompson *et al.*, 1995; Liu *et al.*, 2000). A surprising result to emerge from this study was this monovalent immunotoxin, DT390-sFv, in addition to DT389-sFv, had a 22-fold loss of affinity relative to the parental sFv. This finding was unexpected because of the equal *in vitro* potencies of the monovalent immunotoxins relative the bivalent chemical conjugate. From these results, it can be concluded that the recombinant monovalent immunotoxin possesses highly efficient intoxication mechanisms downstream of the binding step that are able to compensate for poor binding. We reasoned that increasing the affinity of the monovalent recombinant immunotoxin by providing divalent binding would increase its potency.

Our first attempts to increase the affinity involved the generation of divalent constructs including minibodies. Minibodies, first described by Hu *et al.* (Hu *et al.*, 1996), use a single interactive heavy chain domain to stabilize a disulfide dimerized sFv construct such as (sFv-H-γCH3-h)2. We also did this to a previously undescribed minibody utilizing the μCH2 domain to provide the stabilizing interaction and the interchain disulfide bond. These divalent constructs had identical affinity when compared with the native antibody, UCHT1. However, addition of DT390 moeities to the N-termini of the divalent minibodies drastically reduced their binding and failed to show an increase in binding over DT390sFv. In addition, their potency was almost non-existent. Because the addition of a single toxin moiety to the UCHT1 antibody decreased its affinity 3-fold, we suspected that the toxin moiety was providing steric inhibition to the Fv binding domain. If this was the case, positional effects might be important. In fact, addition of the human serum albumin sequence at the N-terminus of the sFv lowered the relative binding 35-fold. This suggested that the N-terminus of the sFv was sensitive to the presence of large foreign protein domains resulting in a reduction of sFv binding affinity. The mechanism could be steric inhibition or, alternatively, the additional moiety could alter overall protein folding, thereby affecting bioactivity (Martsev *et al.*, 2000).

A single-chain construct consisting of two tandem sFv domains provided a possible way to achieve bivalent binding and to minimize steric interactions at the C-terminal sFv. The divalent sFv, bisFv, had binding values slightly better than the native antibody. Addition of the DT390 moiety to the bisFv, A-dmDT390-bisFv (G4S), showed increased relative binding by 6–7-fold over the A-dmDT390-sFv. Moreover, the divalent construct had a 13-fold increase in *in vitro* toxicity compared with the monovalent immunotoxin and the chemical conjugate, UCHT1–CRM9. A bivalent construct utilizing an inter-sFv linker based on Mallender and Vos (Mallender and Voss, 1994) was only as toxic as the monovalent DT390-sFv (data not shown). This implies that the folding of the bivalent construct is an important variable in immunotoxin design. The DT390-bisFv (G4S) single-chain divalent immunotoxin represents a very significant increase in potency over previously described anti-CD3 immunotoxins and this increase in potency is due to an increase in binding to the CD3ε epitope.

The 10-fold enhanced potency of the divalent recombinant immunotoxin relative to the monovalent immunotoxin observed in Jurkat cells was also seen in spleen T cell depletion in the tgε600$^{+/-}$ mice that express human CD3ε. At the highest dose tested, 1026 pmol/kg, the fractional T cell depletion was 0.995 by the probit model. This dose was associated with significant immunosuppressive effects in allograft survival studies (Weetall *et al.*, personal communication). The enhanced potency of the divalent construct was 3–4-fold greater in the comparison of lymph nodes. This enhancement was within the range of the individual *in vitro* assays. Differences between lymph node and spleen data may reflect systematic experimental errors rather than real compartmental differences. However, one could expect a greater potency enhancement *in vivo* compared with *in vitro* owing to decreased renal clearance of the higher molecular weight divalent immunotoxin. Vallera *et al.* reported that a similar recombinant anti-murine anti-CD3 monovalent immunotoxin utilizing DT390 induces renal toxicity (Vallera *et al.*, 2000), presumably by renal filtration and renal tubular reabsorption of this relatively low-MW immunotoxin. Renal toxicity was reduced by adding a C-terminal cysteine that generated a disulfide dimer immunotoxin and thus permitted higher *in vivo* dosing. However, this dimer did not exhibit any increase in *in vitro* potency. We suggest that this result was due to steric hindrance generated by the two DT390 moieties. The configuration of one DT390 moiety followed by a bisFv (G4S) moiety appears to attenuate the steric effect of a single N-terminal DT390 moiety on sFv binding and offers increased *in vivo* and *in vitro* potency over all pre-existing anti-CD3 immunotoxin constructs.

In summary, this report delineates the optimization of an anti-CD3 immunotoxin from the parental chemically conjugated immunotoxin through a monovalent recombinant immunotoxin to a divalent single-chain immunotoxin, A-dmDT390-bisFv (G4S). The parental chemically conjugated anti-human CD3 immunotoxin has a demonstrated *in vivo* activity against leukemic T cells, reducing these cells 3 logs at a tolerable dose in a xenograft model (Neville *et al.*, 1992). The chemically conjugated anti-rhesus analog can transiently deplete 2 logs of resting lymph node T cells in monkeys, a process that can induce long-term allograft transplantation tolerance (Huang *et al.*, 2000; Thomas *et al.*, 2000). The availability of the optimized construct with a 13-fold increase in potency suggests that this reagent will be efficacious *in vivo* against human CD3$^+$ T cell leukemia/lymphoma. The fractional resting T cell depletion of 0.995 in the tgε600$^{+/-}$ mouse at 1026 pmol/kg is noteworthy because this dose of DT389-IL-2, another truncated DT immunotoxin, is well tolerated clinically (Saleh *et al.*, 1998). This suggests that the optimization achieved with A-dmDT390-bisFv (G4S) may have resulted in a therapeutic window that will permit the use of T cell depletion tolerance protocols in human transplantation.

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Exhibit B

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